ASPECTS OF MERISTEM CULTURE IN THE CATTLEYA ALLIANCE (ORCHIDACEAE)

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INTRODUCTION

Since most modern orchid hybrids are extremely heterozygous, it is difficult to obtain large numbers of desirable clones from seedling populations. In addition, current asexual propagation techniques yield only a few plants of a clone per year, demonstrating the need for improved methods of asexual propagation.

Morel (1960) noted the possibilities for clonal increase when meristem culture techniques were applied to Cymbidium clones. Wimber (1963) verified the hypothesis and suggested modifications of previous methods for more rapid multiplication of Cymbidium cultivars. However, Morel (1964) found that Cattleya, perhaps the genus best known and most demanded by consumers, was more difficult to propagate by meristem culture than were other selected genera.

Hence, the purpose of this investigation is to devise a technique for aseptic culture of meristem explants from representatives of the Cattleya alliance and to define a medium which can support growth of such explants.

REVIEW OF LITERATURE

Historically, meristem culture has been used to rid virus infected stocks of various taxa from their disease incitant. Morel and Martin (1955) introduced the use of meristem culture as a means of procuring virus-free plants of carnation and dahlia. Their successes provided the impetus for further efforts in various laboratories, and Quak (1957) also described a method of aseptically removing the apical meristems along with some leaf primordia from carnations to obtain virus-free stocks. Kassanis (1957) applied the technique for use in obtaining virus-free clones of various infected potato varieties, while Manzer (1959) worked specifically on potato varieties infected with virus X. Internal cork virus and latent A virus were eliminated from sweet potato (Nielson, 1960) and strawberry (Belkengren and Miller, 1962) respectively, by application of similar procedures. The responses of carnation to meristem culture were considered by Phillips and Matthews (1964). In all cases reported above, explants of apical tissues gave rise to single plantlets.

However, Morel (1960), in attempting to free Cymbidium clones from Cymbidium mosaic virus by meristem culture, found that explanted tissue became swollen and rounded, forming a "bulblet" which resembled a protocorm. These protocorm-like bodies produced rhizoids on the periphery and a small leaf at the summit; each subsequently grew in a manner not unlike seedlings. In fact, a single explant produced several plantlets, intimating clonal multiplication as a consequence.

In the embryology of orchids, protocorms are oval bodies which result from several mitotic divisions of the zygote and succeeding cells. These bodies lack vascularization and are comprised of two intergrading cell regions; one is parenchymatous while the other is meristematic, the

latter giving rise to the shoot apex. The protocorm-like bodies produced from explants morphologically resemble true protocorms; yet the presence of vascular elements makes them distinct.

Wimber (1963) elaborated the methods for Cymbidium and included agitation of explanted tissue in liquid medium as a means of inhibiting shoot formation with the consequence of increasing the growth rate of protocorm-like bodies arising from explants. Multiplication of clonal material was accomplished by aseptic division of tissue masses grown in liquid culture with subsequent transfer of sections to a solid medium. The undisturbed tissue produced plantlets in 2½ months thereafter, or usually within 4-6 months after the original isolation.

By 1964, Morel had flowered plants of Cymbidium, Miltonia, and Phaius, which had been propagated by meristem culture 5-6 years earlier. Phalaenopsis and Vanda gave very poor or entirely negative results; trials were being made with Cattleya. Later reports (Morel, 1965a, 1965b) indicated that Cattleya, Odontoglossum, Lycaste, and Dendrobium explants also gave rise to protocorm-like bodies, and suggested use of indoleacetic acid (IAA) or naphthalene acetic acid (NAA) as media supplements at one part per million (1 ppm) concentration. Coconut water and pineapple juice each at 10% concentration were also suggested as separate additives for Morel's formula.

Wimber (1965) recognized that leaf tissue of some aseptically grown Cymbidium plantlets in liquid medium could produce protocorm-like bodies when constantly buffeted against the sides of glass flasks rotating at 200 revolutions per minute (rpm). No similar findings have been reported, although a detailed description of technique has been outlined (Sagawa et al., 1966). Wimber (1963, 1965) reported that plantlets resulting

from meristem cultures were cytologically no different from parent tissue, while Morel (1960) found that virus infected Cymbidiums propagated by meristem culture were freed from the virus.

MATERIALS AND METHODS

Plants of the Cattleya alliance used in this study were from sources in Honolulu, Hawaii, as indicated below.

	University of Hawaii	Foster Botanical Garden	Oscar Kirsch Orchids
Brassavola nodosa	+	+	
Broughtonia sanguinea		+	+
Cattleya bowringiana	+	+	
C. labiata var. *percivaliana*	+		
C. labiata var. *trianaei*		+	+
C. skinneri		+	
Cattleya spp.	+		
Caularthron (*Diacrium*) *bicornutum*		+	
Epidendrum atropurpureum	+		
E. brassavolae		+	
E. cochleatum	+	+	
E. conopseum	+		
E. fragrans	+	+	
Laelia anceps	+		+
L. flava	+		+
L. pumila	+		
L. purpurata		+	+
Rhyncolaelia (*Brassavola*) *digbyana*	+	+	+
Rhyncolaelia (*Brassavola*) *glauca*	+	+	
Schomburgkia superbiens			+

Vegetative shoots (Fig. 1), ranging from 1 to 8 cm, were used as a source of explants. Explants were cultured using the following media: 1) Vacin and Went's medium modified with 25% (by volume) coconut water (Vacin and Went, 1949); 2) Iwanaga's medium modified from Knudson C (Knudson, 1946) by the addition of 0.05 g/liter ferric phosphate, 1.0 g/liter yeast extract, 3.0 g/liter peptone, and 25% (by volume) coconut water; 3) Morel's medium (Morel, 1965a, 1965b) to which was added 1 ppm naphthalene acetic acid (NAA) and 10% (by volume) coconut water. In all cases, pH was adjusted to 5.0-5.2, using 1N HCl, 10% H_3PO_4, or 1N NaOH. Flasks (50 ml) containing either 5 ml liquid medium or 20 ml solid medium (prepared with 0.8% Difco-Bacto agar) were autoclaved for 10-12 minutes at 15 pounds pressure. Sterile petri dishes were prepared similarly.

Aseptic manipulations were accomplished on the counter top in a transfer room serviced by a Westinghouse Precipitron Type PH122 air conditioning system. Work and storage surfaces therein were illuminated with a 15 watt General Electric Germicidal C15T8 lamp, when not in use. Instruments used were sterilized in absolute ethyl alcohol.

Cultures were stored and maintained at constant temperature 26 $\pm$ 3 C with continuous illumination of about 100-180 foot candles from General Electric Power Groove fluorescent tubes (F48PG17-CW). A New Brunswick Model V rotary action shaker operating at 160 rpm was used to agitate liquid cultures.

Sterilizing solutions were prepared from CLOROX and ZEPHIRAN CHLORIDE with concentrations determined on a volume basis. CLOROX solutions of 20%, 10%, and 5% concentration were prepared by diluting the stock with distilled water whereas a 1% solution was made using one part 5% CLOROX to 4 parts sterile distilled water. ZEPHIRAN CHLORIDE was prepared from

a 17% concentrate, using 29 ml concentrate in 3756 ml distilled water for a final 1:750 aqueous solution. For wetting agent, a few drops of American Cyanamid's SUR-TEN were added to CLOROX concentrations indicated in Table I.

Sequential treatments used in CLOROX are summarized in Table I. Stage I is a vegetative shoot which has been removed from the pseudobulb. Stage II is obtained by removing leaves from the shoot until two lateral buds are exposed, while Stage III exists when all leaves but one are removed. Stages in ZEPHIRAN CHLORIDE are not noted since all dissections were effected in a liquid system; however, manipulations in the liquid require 10-12 minutes.

TABLE I. MERISTEM CULTURE STERILIZATION SCHEDULE IN MINUTES

	TREATMENT I			TREATMENT II			TREATMENT III	TREATMENT IV
	CLOROX			CLOROX + SUR-TEN			CLOROX	ZEPHIRAN CHLORIDE
	10%	5%	1%	10%	5%	1%	20%	1:750
Stage I	15	-	-	15	-	-	5	10-12 min. liquid system
Stage II	-	5-8	-	-	5-8	-	10	
Stage III	-	-	3	-	-	3		

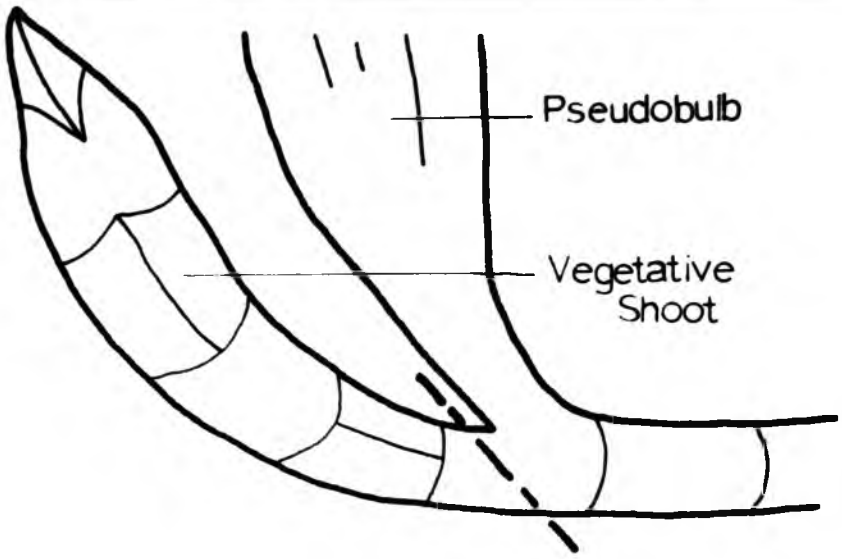

Figure 1. Cattleya Pseudobulb and Vegetative Shoot.

Dotted line indicates point of removal of vegetative shoot, 1-8 cm in length, from the pseudobulb.

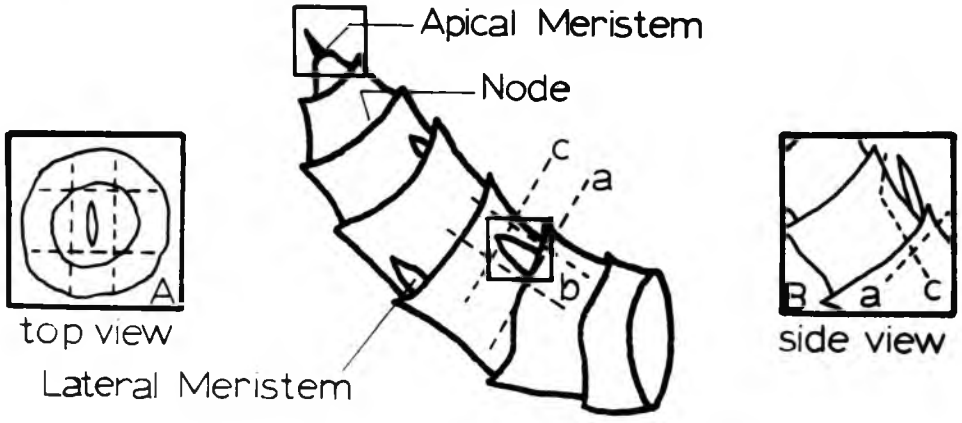

Figure 2. Dissection of Meristems from Vegetative Shoot in Stage III.

Lateral meristems were delimited by incising below the node (a) and adjacent to the bud on either side (b). Final removal depended on a cut 1.0 mm behind the bud, beginning at c (Inset B). Apical meristem was excised as a 1.0 mm cube, as indicated by Inset A.

RESULTS

Use of sterilization Treatments I, II, and IV (Table I) resulted in contamination levels exceeding 50%. Treatment III, which involved sterilizing Stages I and II for 5 and 10 minutes respectively, effectively reduced the number of contaminated cultures to less than 24%. Contamination was usually bacterial although fungal sources were responsible in a few cases.

The technique devised for use in this study was as follows. Lateral meristems were delimited after removing 1-2 embryonic leaves by incising just below the nodal origin and immediately adjacent to either side of the remaining leaves (Fig. 2). The explant was finally removed by cutting 1.0 mm behind the bud itself. Apical meristems were excised by proceeding to Stage III without intervening CLOROX treatment, and removing 1.0 cubic mm of tissue; volume of all explants ranged from 1.0 to 2.0 cubic mm. Explants were aseptically placed on water saturated Whatman No. 1 filter paper in sterile petri dishes before being transferred to culture flasks containing liquid medium.

Liquid cultured explants remained on the shaker 2-4 weeks before being sectioned in an arbitrary plane and transferred to equivalent solid medium. Depending on the development of the explant, sectioning was either transverse or longitudinal with respect to the growth axis. Once proliferation (formation of protocorm-like bodies) had begun, tissue masses were sub-cultured by division in sterile petri dishes without regard for individual entities. Sections of tissue were then placed with cut surfaces in contact with the solid medium. Occasional divisions were returned to liquid culture on the shaker for 7-10 days, resulting in as much as a five-fold increase in tissue volume. Tissues were kept in a

proliferating condition by division and sub-culture at two week intervals. Undisturbed proliferating tissue produced within 6-8 weeks, well-rooted plantlets (Fig. 3-4) ready for removal from the aseptic flask.

Growth was similar from 10 of 12 successful cultures including: two Cattleya bowringiana, C. skinnerii, six Cattleya spp. and Schomburgkia superbiens. Sectioned explants, on solid medium following shake-culture, became swollen with pronounced roughening of surface (Fig. 3-1) after 4-10 weeks (Table II). After such tissues were divided, further growth resulted in numerous clumps of protocorm-like bodies in a mass surrounding the division of the original explant (Fig. 3-2). If left undisturbed for 3-4 weeks, growing points gave rise to plantlets without roots or rhizoids (Fig. 3-3). Minute protocorm-like bodies about the plantlet were removed and sectioned to prolong the culture indefinitely. In two excepted instances, single protocorm-like bodies arose on explants of Epidendrum conopseum and Cattleya spp. (Table II, 4); sub-culture of the single body in either case was unsuccessful. Portions of the proliferating tissue from C. skinnerii lacked chlorophyll in early stages of growth prior to sub-culture.

Ten of 12 successful cultures were produced on Vacin and Went's medium (Table III), while the remaining two were cultured on Morel's medium. No cultures on Iwanaga's medium produced protocorm-like bodies. Eight successful cultures arose from explants of lateral origin while the remaining four were of apical origin.

Differences in time elapsed between the original culturing and initial production of protocorm-like bodies were evident (Table II). In Cattleya spp., periods ranged from 45-83 days; periods for successful

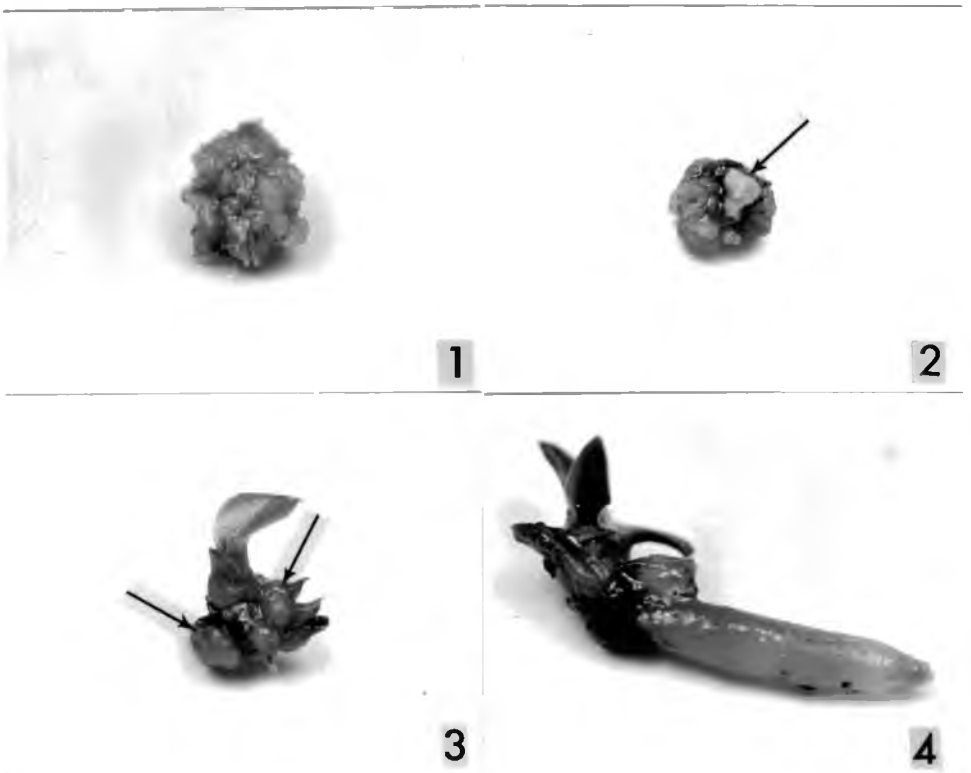

Figure 3. Sequential Development of Protocorm-like Bodies from Tissue Explants of Cattleya.

1. Roughened surface of original explant is caused by formation of protocorm-like bodies.

2. Protocorm-like bodies surround a piece of the initial explant (arrow).

3. Plantlet without roots or rhizoids is encircled at base with protocorm-like bodies (arrows).

4. Plantlet with root which developed 6-8 weeks after sub-culture is ready for greenhouse culture.

cultures of C. skinneri, C. bowringiana (Nos. 1 and 2), E. conopseum, and Schomburgkia superbiens were 85, 56 and 95, 52, and 67 days respectively.

Some plant materials were noticeably less tolerant of the sterilizing agent used than were others. After Stage I, Treatment III, explants of Laelia anceps, Broughtonia sanguinea (with exceptions as indicated in Table III), and all tissues of representatives in the genus Epidendrum, were considerably less turgid than at the outset. Cultured explants often failed to grow and consequently were discarded within the first week after excision. Numerous cultures of other plants in all media failed to grow and were discarded after a 3-4 week period of shaking (Table III).

TABLE II. OUTLINE OF SUCCESSFUL CULTURES FROM MERISTEM EXPLANTS

	MATERIAL	POSITION OF MERISTEM	MEDIUM*	TIME (days) ON SHAKER	TIME (days) ON SOLID MEDIUM BEFORE GROWTH	TOTAL PERIOD (days)
1.	Cattleya bowringiana	apical	M	25	70	95
2.	" "	lateral	V	14	42	56
3.	Cattleya skinnerii	lateral	M	29	56	85
4.	Cattleya spp.	apical	V	21	52	73
5.	"	lateral	V	21	52	73
6.	"	lateral	V	21	24	45
7.	"	lateral	V	20	29	49
8.	"	lateral	V	20	29	49
9.	"	lateral	V	20	63	83
10.	"	lateral	V	14	42	56
11.	Epidendrum conopseum	apical	V	24	28	52
12.	Schomburgkia superbiens	apical	V	35	32	67

* V = Vacin and Went's medium
M = Morel's medium

TABLE III. SUMMARY OF MERISTEM CULTURES

	Vacin & Went					Morel					Iwanaga				
	Total Cultures	Contaminants	No Growth	Sectioned and Retained	Protocorm-like Formation	Total Cultures	Contaminants	No Growth	Sectioned and Retained	Protocorm-like Formation	Total Cultures	Contaminants	No Growth	Sectioned and Retained	Protocorm-like Formation
Brassavola nodosa	10	1	8	1	-	4	-	4	-	-	10	-	10	-	-
Broughtonia sanguinea	8	-	7	1	-	7	4	1	2	-	-	-	-	-	-
Cattleya bowringiana	15	1	3	11	1	9	2	-	7	1	7	2	2	3	-
C. labiata var. percivaliana	-	-	-	-	-	-	-	-	-	-	3	-	1	2	-
C. labiata var. trianaei	4	-	-	4	-	9	1	5	3	-	6	-	2	4	-
C. skinnerii	5	-	-	5	-	3	1	-	2	1	5	-	4	1	-
Cattleya spp.	33	11	4	18	7	6	1	4	1	-	5	1	1	3	-
Caularthron bicornutum	2	2	-	-	-	1	-	1	-	-	-	-	-	-	-
Epidendrum atropurpureum	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
E. brassavolae	2	1	-	1	-	1	-	1	-	-	1	-	-	1	-
E. cochleatum	5	4	-	1	-	1	-	-	1	-	2	-	2	-	-

TABLE III. (Continued) SUMMARY OF MERISTEM CULTURES

	Vacin & Went					Morel					Iwanaga				
	Total Cultures	Contaminants	No Growth	Sectioned and Retained	Protocorm-like Formation	Total Cultures	Contaminants	No Growth	Sectioned and Retained	Protocorm-like Formation	Total Cultures	Contaminants	No Growth	Sectioned and Retained	Protocorm-like Formation
E. conopseum	1	-	-	1	1	-	-	-	-	-	-	-	-	-	-
E. fragrans	6	4	-	2	-	4	1	1	2	-	5	5	-	-	-
Laelia anceps	3	-	3	-	-	2	-	2	-	-	2	-	-	2	-
L. flava	1	-	1	-	-	-	-	-	-	-	2	-	-	2	-
L. pumila	4	2	1	1	-	6	2	3	1	-	3	1	1	1	-
L. purpurata	3	-	1	2	-	6	1	2	3	-	5	-	5	-	-
Rhyncolaelia digbyana	5	2	-	3	-	1	1	-	-	-	5	1	4	-	-
Rhyncolaelia glauca	15	3	2	10	-	2	-	-	2	-	-	-	-	-	-
Schomburgkia superbiens	3	-	1	2	1	3	-	-	3	-	-	-	-	-	-
Totals	126				10	65				2	61				0

DISCUSSION

Various tissue culture studies have shown that coconut water has a growth stimulating effect on callus, suggesting its use as a medium supplement for the current investigation. Moreover, the growth promoting activity of liquid endosperms and endosperm extracts from several plants in general has been recognized for some time. Steward and Shantz (1959) summarized use of endosperm supplements in tissue culture work, noting that liquid endosperm from corn (Zea), extract of female gametophyte in Ginkgo, and fluid from the embryo sac of horse-chestnut (Aesculus) all showed growth inducing activity. A few years earlier, such activity was reported (Steward et al., 1952) from coconut water, and Shantz and Steward (1952) proceeded to fractionate the liquid endosperm into nonspecific (reduced nitrogen), neutral, and active fractions. Using a bioassay technique with carrot tissue (Steward et al., 1952), it was determined that casein hydrolysate could replace the so-called non-specific fraction to achieve maximum relative growth when combined with the active fraction. Moreover, the neutral fraction singly produced little response until used with the active fraction. Compounds A, B, and C of the active portion were distinguished (Shantz and Steward, 1952) with subsequent identification of compound A as 1,3-diphenylurea (Shantz and Steward, 1955). The neutral fraction was comprised principally of the hexitols, d-sorbitol, myo-inositol, and scyllo-inositol (Pollard et al., 1959). In addition to the successes in tissue culture of carrot explants, similar stimulation has been recorded for cultures of Jerusalem artichoke, tobacco, salsify, grapevine, and Parthenocissus (Duhament, 1959).

The idea that naphthalene acetic acid (NAA), yeast extract, and peptone might promote growth of explanted orchid tissue was deduced from

the findings of various investigations in which these materials were used for similar purposes. NAA was found to be superior to indoleacetic acid (IAA) in growth promotion of Solanum tuberosa callus (Lingappa, 1957). In fact, the best growth was achieved when NAA and coconut water were used in combination. Morel (1965a, 1965b) found 1.0 ppm NAA to have a stimulatory effect on Cymbidium cultures, and that finding was confirmed in this laboratory. Yeast extracts have been used for tissue cultures of tobacco (Sandstedt and Skoog, 1960) and Osmunda apical meristems (Sussex, 1963). Further, Sussex and Steeves (1953) grew gametophytes of Pteridium aquilinum var. latiusculum on Knudson's medium including 0.5 g/liter yeast extract, but concluded that growth factors were not needed for growth of the tissue. Knudson (1922, 1950) used yeast preparations to improve asymbiotic orchid seed germination. Peptone as a supplementary growth factor has been used for germination of Cypripedium seed (Withner, 1953) as well as other orchid seeds (Hegarty, 1955; Curtis, 1947).

Twenty-five percent coconut water (from green coconuts) was arbitrarily chosen as the concentration of the liquid endosperm to be used with the Vacin and Went medium (1949) which was reported to undergo little pH change after autoclaving and storage. Using the same medium containing 25% coconut water, Sagawa and Niimoto (1962) aseptically cultured and grew Phalaenopsis embryos. Earlier, orchid seeds were reported to have been germinated on media supplemented with coconut water (Hegarty, 1955) with success. For carrot tissue cultures, the optimum growth was achieved with 10-15% coconut water added to the medium, although it was suggested that growth could occur in 100% coconut water (Steward et al., 1952). Later Caplin (1956) used 15% coconut water for a bioassay system. Even so, variations in the activity of the additive were possible depending

on the source (Caplin and Steward, 1952). Yet, Steward and Caplin (1952b) showed that the fluid endosperm was active at all stages of development, and allowing for possible variability, no significant effect of age on activity was detected.

Modification of Knudson's C medium (Knudson, 1946) by the addition of ferric phosphate (Knudson, 1922), yeast extract, peptone, and 25% coconut water resulted in Iwanaga's formula. The latter three substances were all known to have growth promoting qualities when used singly for various purposes previously described; ferric phosphate was a carry-over from Knudson's B nutrient solution. However, there seemed to be no reason to use all the above materials as supplements except that limited success had been reported for Cymbidium and Cattleya meristem cultures in E. T. Iwanaga's laboratory in Honolulu (personal communication).

Morel's medium (Morel, 1965a, 1965b) was derived from a formulation used for potato meristem cultures. NAA and coconut water, 1.0 ppm and 10% respectively, were supplemented in that ratio since coconut water was reported to already contain some NAA (Wiggans, 1954). Morel reported success in propagating Lycaste, Cymbidium, Cattleya, Dendrobium, Miltonia, and Phaius on a similarly concocted medium using meristem culture techniques (Morel, 1965a).

One other additive tried for use with Morel's medium was unsweetened, canned pineapple juice (Morel, 1965b). The composition of fresh juice as described by Gortner and Singleton (1965) closely resembled that of coconut water (Tulecke et al., 1961). However, no growth resulted from explants on the medium containing 10% canned pineapple juice. Since proteases and peroxidases present in fresh juice were inactivated by the heat of sterilization, this suggested that either an artifact of

autoclaving or processing, or some constituent present in excess or deficit in the supplement, resulted in the decline of the cultures. Further use of pineapple juice was omitted.

Two explants produced protocorm-like bodies while growing on Morel's medium. Growth of the C. skinnerii tissue was peculiar in that portions of the mass were devoid of chlorophyll. Since the only other culture (C. bowringiana-1) to produce protocorm-like bodies on the medium had a faint sign of chlorophyll throughout the mass, the evidence was not sufficient to designate the effect as one of the medium. Furthermore, Morel made no report of similar findings in his work, although Wimber (1965) suggested that cultures on his medium were often chlorophylless. Hence, more trials would be necessary to make an accurate evaluation.

Artichoke tuber tissue growing on coconut water medium accumulates a greater concentration of soluble nitrogen compounds than do carrot or potato tissues, suggesting some limitation on maximum protein synthesis which is not wholly satisfied by coconut water and/or NAA (Steward et al., 1960). If this theory is extended to the situation on Morel's medium, additional modifications would be necessary, perhaps using a coconut water-2,4-dichlorophenoxyacetic acid (2,4-D) combination.

Iwanaga's medium contained such a high level of several growth factors, that the lack of success in supporting tissue cultures was not surprising. Caplin (1956) described depressed growth of carrot tissues when IAA and coconut milk factor (CMF) were present in excess of the optimum 0.1 mg/liter and 15% respectively. Extracts of tumor tissue incited on Kalanchoe by Agrobacterium tumefaciens stimulated growth of carrot tissue when used in an optimal concentration ratio with coconut water, but inhibited growth when used at higher rates (Steward et al., 1955). Perhaps

trials with much reduced concentrations of additives to the basic medium would be rewarding.

Vacin and Went's formula with 25% coconut water proved to be the most satisfactory in this work, with 10 of 12 successful cultures being produced on it. Even so, manipulation of coconut water concentrations might result in further successes, since again Caplin and Steward (1952) showed that an excess of coconut water depressed growth of carrot tissue and that such inhibition was caused by the active fraction.

Choices of media to be compared in this investigation were made for specific reasons, enumerated above. On the other hand, White's basal culture medium (White, 1954) seemed to be too complex for use in Cymbidium tissue cultures; explants which failed to grow on this medium responded after transfer to Knudson's C formula (Knudson, 1946) with production of protocorm-like bodies. Furthermore, trials with Cattleya showed that the 25% coconut water supplemented and unsupplemented Knudson's C medium were inefficacious, in direct contrast to the outcome with Cymbidium. Hence, neither of these formulations were included.

Within each apex, Stant (1954) envisages a central corpus of cells dividing in diverse directions and a tunica of one to several layers of smaller cells in anticlinal division; both are initially derived from the apical initials which are larger and divide less frequently than the adjacent cells. The corpus consists of rib (or central) and flank (or peripheral) meristems. The former is a zone wherein arise files of partly vacuolated cells produced by the transverse formation of new walls; the flank meristem surrounds the preceding zone and takes part in initiation of new leaf primordia. Even though the above is a general description of the apex, one must consider that apices of different genera

can vary in the proportion of tissue which may be ascribed to the separate zones.

Although the source of the meristems cultured from the vegetative shoot was apparently insignificant since 4 apical and 8 lateral explants produced protocorm-like bodies, the state of development of vegetative shoot was of paramount importance. Protocorm-like bodies arose not from a particular site on the node, but rather from a region more accurately characterized as an intercalary meristem (Esau, 1960). The phenomenon was noticed initially in Cymbidium cultures in this laboratory, and the same situation seemed to prevail in cultures of the Cattleya alliance. Further, Stant (1954) indicated that in Narcissus, the absence of development in the internode was associated with a very low proportion of rib meristem. In Cattleya Trimos, little rib meristem was present in growths of the size ultimately used in these studies (Shushan, 1959). Hence, maximization of intercalary meristem potential was achieved with growths 1-8 cm in length. Since lateral buds were inactive in that apical dominance exists in the Cattleya as described by Shushan (1959), there was also a maximization of intercalary meristem potential when such buds were removed from the influence of the shoot's apical meristem. Once internodal elongation was complete, i.e. vegetative growth had ceased for the branch (often termed the pseudobulb or growth), intercalary meristem activity was at a minimum.

Even though vegetative shoots 1-8 cm in length have served as a source of explants in this study, it would be interesting to determine if responses of explants taken from shoots of greater or lesser dimensions would be different. In a small vegetative shoot, the potential

of intercalary meristem activity would be greater, and growth would more likely result from such tissue than from that taken from larger shoots.

The fact that Wimber (1965) was able to obtain protocorm-like bodies from a Cymbidium leaf on a young plant must not be overlooked even though such an occurrence is in direct opposition to the previous argument. Explanation lies most likely in the fact that parenchyma cells by definition have the capacity for division and growth, and that production of protocorm-like bodies resulted after continuous abrasion - exogenous stimulation - of parenchyma cells in the leaf tissue.

Furthermore, Sussex (1963) suggested that development of growth centers ultimately derived from apical meristems may be modified by an exogenous stimulus such as photoperiod. Potato callus cultures failed to grow with an optimum level of coconut water in the basal medium until 2,4-D, another exogenous stimulus, had been added (Steward and Caplin, 1952a). Such response implied that some growth inhibitor was inherently present in potato parenchyma, and that by supplying 2,4-D, the effects of the inhibitor were overcome or antimetabolized (Steward and Caplin, 1952a), thus allowing coconut water to express its growth promoting influence.

In discussing growth, the definition has been presupposed to include cell division as well as enlargement. Continuing with the previous argument, the necessity for a specific compound to induce cell division (Steward and Shantz, 1959) has been demonstrated. In carrot tissue, aromatic substitution in the 4 position of phenoxy acids was conducive to cell division, while a substitution in the 2 position encouraged enlargement. Hence, an optical enantiomorph of a compound promoting growth may be completely antagonistic in that sense.

Since Cymbidium tissues are apparently more easily cultured by these techniques, there must be a greater degree of autonomy in Cymbidium explants than in those of Cattleya and its alliance. Such a conclusion dictates a need by the latter tissues for an exogenous stimulus for growth whereas in Cymbidium the factor(s) is already present within the explant or in the media tried for both alliances. As an alternative possibility, some moiety in the media may be present in excess, causing an inhibition of growth as previously proposed.

Since Cattleya explants have not resulted in callus cultures, perhaps the use of some growth regulator might induce cell division with another such compound accounting for subsequent enlargement. Although Steward and Shantz (1959) elicited a minimum of growth promotion by adding gibberellins to carrot callus cultures, the addition of kinetin resulted in a very significant increase in cell numbers and weight (Steward et al., 1960). Further, evidence was found indicating gibberellins may be antagonistic with the growth promotional activity of coconut water (Steward and Shantz, 1959; Steward et al., 1960); kinetin and kinetin-like compounds definitely had a promotional effect on carrot calluses in the presence of IAA and absence of coconut water.

Steward and Shantz (1959) assume that once cells have received the stimuli for division, the daughter cells should remain autotrophic in that respect. In such a system, cultures could be maintained indefinitely without mechanical disturbance. However, in Cattleya, orderly growth probably similar to that proposed by the same authors, results from a stimulus arising from within the cell mass. Internal cells may lose their contents, lignify, and differentiate, forming tracheid-like elements and creating a stimulus which emanates from the center to induce

orderly cell division throughout. By regular sub-culture, such systems are not permitted to develop; hence, juvenility is maintained. Growth of small tissue masses in agitated liquid culture proceeds at an increasing rate exponentially and finally at a decreasing rate as the mass enlarges and the relative surface area is decreased (Steward et al., 1952), thereby accounting for a five-fold increase in tissue volume in a 10-day period following sub-culture in liquid medium.

The sterilization methods finally employed for this investigation seemed to be satisfactory. Some damage occurs on the explants during excision and sterilization, yet no studies have been made to determine the extent of such impairment and its effect on success of cultures. In carrot liquid cultures, early reduction in growth after explanting indicated presence of a specific inhibitor, or "staling product," which may have originated from the tissue cultures (Caplin and Steward, 1952). Such a possibility exists in the present work, suggesting that repeated transfers to fresh medium following initial explanting would minimize this factor.

In view of the evidence, it seems that Cattleya explants require agitation to maximize the response from growth centers developed within, after the tissue is transferred to solid medium. Rotation of explants in liquid medium aids aeration, providing for full activity in respiration, protein syntheses, and salt intake. Further, the use of agitation in liquid cultures to minimize the response to gravitational stimuli has been described for carrot tissue cultures (Steward et al., 1952, 1960). Wimber (1963) reported that shaking of Cymbidium explants inhibited shoot formation from proliferating tissues. Morel (1965b) was successful in growing Cattleya explants on solid medium, but found that best results

were obtained by use of an agitated liquid medium. Cymbidium explants on the other hand, can be grown directly on solid medium without such shaking (Morel, 1960, 1964, 1965b; Wimber, 1963, 1965; Sagawa et al., 1966). In the current work, all cultures were agitated since preliminary attempts to grow the explants on solid medium were unsuccessful.

Protocorm-like bodies formed on explants of Cattleya alliance in this study, lack rhizoid development. Morel (1965b) noted that some rhizoids were formed on the protocorm-like bodies of Cattleya, but not to the extent that they were in Cymbidiums. Moreover, Morel (1960, 1964, 1965a, 1965b) as well as Wimber (1963) and Sagawa (personal communication) have noticed rhizoid formation on protocorm-like bodies of Cymbidium. Burgeff (1936) indicated that protocorms developing from seeds of Cymbidium and Cattleya have papillae or rhizoids. Shushan (1959) also observed rhizoids in development of protocorms from seeds of Cattleya Trimos. The above evidence further emphasizes the differences which exist in the development of protocorm-like bodies from the two genera mentioned. Cattleya explants probably require added metabolites in order to produce protocorm-like bodies.

There were differences in the total period from excision to production of protocorm-like bodies for most of the successful cultures (Table II). Steward (et al., 1952) indicated that carrot callus was formed after a lag period and that the period varied even for explants from the same carrot. Explants numbered 7, 8, and 9 (Table II) were all explants from the same vegetative shoot of Cattleya spp. However, as shown by the tabulation, there were also differences in responses of explants 7 and 8 vs. number 9. Such an occurrence may be explained on the basis that the lateral buds from which the explants were made, probably were

in different metabolic or physiologic states due to their relative positions on the vegetative shoot. Other differences may have been due to genetic variation in the experimental material. Caplin and Steward (1952) recognized this variation to be present in the carrot tissue, and attributed it to inherent morphology.

Whereas other flowering plants including carnation (Quak, 1957; Phillips and Matthews, 1964; Morel and Martin, 1955), sweet potato (Nielson, 1960), potato (Manzer, 1959; Kassanis, 1957), strawberry (Belkengren and Miller, 1962), and dahlia (Morel and Martin, 1955) produce single plantlets from a cultured meristem, orchids produce large numbers of protocorm-like bodies resulting in numerous plantlets from a single explant. It is the phenomenon of proliferation and production of protocorm-like bodies which makes orchids unique in their response. By maintaining a culture producing protocorm-like bodies, large numbers of identical plants can be propagated.

However, propagation of any orchid at will, regardless of genus, may not be a reality for considerable time. To accomplish the ideal, a better understanding of the explant's requirements must be obtained. Numerous alternatives have been proposed in this respect, but apparently the use of coconut water as a nutritive and growth promoting substance provides a useful tool for further progress. Even so, location of a clone in the Cattleya alliance which consistently produces protocorm-like bodies from explanted tissue would provide a means of assaying the various media. With such a system, and allowing for inherent clonal variability, determination of factors which are necessary to promote the production of protocorm-like bodies would be greatly aided.

SUMMARY

The purpose of this investigation was to devise a technique and define a medium for aseptic culture of meristem explants from representatives of the Cattleya alliance.

One cubic millimeter meristem explants were excised from vegetative shoots 1-8 cm in length and sterilized in 20% CLOROX. Explants were cultured on Vacin and Went, Morel, and Iwanaga media; ten of twelve successful cultures were produced on the Vacin and Went medium while two grew on Morel's medium. In all cases, explants were agitated for 2-5 week periods in liquid medium before transfer to solid medium.

Differences between the responses of Cymbidium and Cattleya were noted. Cymbidium was successfully cultured on similar media, more readily than Cattleya. demonstrating the dissimilarity between the two genera in the degree of autonomy within the explant. The use of various media supplements was suggested.

It was determined that coconut water contains substances which promote growth from Cattleya explants. However, it was postulated that either variations in coconut water concentrations or use of other growth regulators as additives to basal media, would be beneficial.

Location of a clone in the alliance which can be readily cultured by these techniques would provide a useful tool for a bioassay system to be employed in evaluating various additives and their effects on this system. Such a tool would contribute much towards understanding growth and development of explanted tissues.

LITERATURE CITED

Belkengren, R. O. and P. W. Miller. 1962. Culture of apical meristems of Fragaria vesca, strawberry, plants as a method of excluding latent A virus. Plant Disease Reporter 46:119-121.

Burgeff, H. 1936. Samenkeimung der Orchideen. Gustav Fischer, Jena, Germany.

Caplin, S. M. 1956. Variability of carrots to auxin, casein hydrolysate, and coconut milk. Amer. Jour. Bot. 43:749-754.

_____ and F. C. Steward. 1952. Investigations on growth and metabolism of plant cells II. Variables affecting growth of tissue explants and the development of a quantitative method using carrot root. Ann. Bot. 16:219-234.

Curtis, J. T. 1947. Studies on nitrogen nutrition of orchids' embryos I. Complex nitrogen sources. Amer. Orchid Soc. Bull. 16:654-660.

Duhament, L. 1959. Cited from Steward and Shantz. 1959.

Esau, K. 1960. Anatomy of Seed Plants. John Wiley and Sons, New York.

Gortner, W. A. and V. L. Singleton. 1965. Chemical and physical development of the pineapple fruit III. Nitrogenous and enzyme constituents. J. Food Sci. 30(1):24-29.

Hegarty, C. P. 1955. Observations on orchid seed germination. Amer. Orchid Soc. Bull. 24:457-464.

Kassanis, B. 1957. The use of tissue cultures to produce virus-free clones from infected potato varieties. Ann. Applied Biol. 45:422-427.

Knudson, L. 1922. Nonsymbiotic germination of orchid seeds. Bot. Gaz. 73:1-25.

_____. 1946. Solution C for orchid seedlings in culture. Am. Orchid Soc. Bull. 15:214-217.

_____. 1950. Germination of seeds of Vanilla. Amer. Jour. Bot. 37:241-247.

Lingappa, Y. 1957. Tissue cultures of Solanum tuberosa and Ipomea pandurata. Amer. Jour. Bot. 44:419-423.

Manzer, F. E. 1959. Potato meristem culture and virus X. Amer. Potato Jour. 36:191-195.

Morel, Georges M. 1960. Producing virus-free Cymbidiums. Amer. Orchid Soc. Bull. 29:495-497.

Stant, Margaret Y. 1954. The shoot apex of some monocotyledons II. Growth organization. Ann. Bot. 18:441-447.

Steward, F. C. and S. M. Caplin. 1952a. Investigations on growth and metabolism of plant cells III. Evidence for growth inhibitors in certain mature tissues. Ann. Bot. 16:476-489.

_____ and _____. 1952b. Investigations on growth and metabolism of plant cells IV. Evidence on the role of coconut milk factor in development. Ann. Bot. 16:491-504.

_____, _____, and F. K. Miller. 1952. Investigations on growth and metabolism of plant cells I. New techniques for the investigation of metabolism, nutrition, and growth in undifferentiated cells. Ann. Bot. 16:57-77.

_____, _____, and E. M. Shantz. 1955. Investigations on growth and metabolism of plant cells V. Tumorous growth in relation to growth factors of the type found in coconut. Ann. Bot. 19:29-47.

_____ and E. M. Shantz. 1959. The chemical regulation of growth (some substances and extracts which induce growth and morphogenesis). Ann. Rev. Plant Physiol. 10:379-404.

_____, _____, J. K. Pollard, M. O. Mapes, and J. Mitra. 1961. Growth induction in explanted cells and tissues: metabolic and morphogenetic manifestations. In Synthesis of Molecular and Cellular Structure. Dorothea Rudnick, ed. Ronald Press, New York. Pp. 193-246.

Sussex, I. M. 1963. The permanence of meristems: developmental organizers or reactors to exogenous stimuli? In Meristems and Differentiation. Brookhaven Symposia in Biology No. 16.

_____ and Taylor Steeves. 1953. Experimental and analytical studies of pteridophytes XXI. Sterile cultures of an abnormal growth from the gametophyte of bracken. Ann. Bot. 17:395-401.

Tulecke, W., L. H. Weinstein, A. Rutner, and H. J. Laurencot, Jr. 1961. The biochemical composition of coconut water (coconut milk) as related to its use in plant tissue culture. Contrib. Boyce Thompson Inst. 21(2):115-128.

Vacin, E. and F. Went. 1949. Some pH changes in nutrient solutions. Bot. Gaz. 110:605-613.

White, P. R. 1954. The Cultivation of Animal and Plant Cells. Ronald Press, New York. 228 p.

Wiggans, S. C. 1954. Growth and organ formation in callus tissue derived from Daucus carota. Amer. Jour. Bot. 41:321-326.

_____. 1964. Tissue culture - a new means of clonal propagation of orchids. Amer. Orchid Soc. Bull. 33:473-478.

_____. 1965a. Eine neue methode erbgleicher vermehrung die kultur von triebspitzen-meristemen. Die Orchideen 16(3):165-176.

_____. 1965b. Clonal propagation of orchids by meristem culture. Cymbidium Soc. News 20(7):3-11.

_____ and C. Martin. 1955. Guerison des plantes atteintes de maladies a virus; par culture de meristemes apicaux. Report of XIV Intern. Hort. Cong., Netherlands. Pp. 303-310.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nielson, L. W. 1960. Elimination of internal cork virus by culturing apical meristems of infected sweet potatoes. Phytopath. 50:840-841.

Niimoto, D. H. and Y. Sagawa. 1962. Ovule development in Phalaenopsis. Caryologia 15:89-97.

Phillips, Douglas J. and Gary J. Matthews. 1964. Growth and development of carnation shoot tips in vitro. Bot. Gaz. 125:7-12.

Pollard, J. K., E. M. Shantz, and F. C. Steward. 1959. Growth promoting activity of coconut milk: nature of non-ionic components. Plant. Physiology 34:(suppl.) viii.

Quak, F. 1957. Meristem culture, combined with heat treatment, in order to obtain virus-free carnation plants. Tijdschr. Plantenziekten 63:13-14.

Sandstedt, R. M. and F. Skoog. 1960. Cited after Murashige and Skoog. 1962.

Sagawa, Y., T. Shoji, and T. Shoji. 1966. Clonal propagation of Cymbidiums through shoot meristem culture. Amer. Orchid Soc. Bull. 35:118-122.

Shantz, E. M. and F. C. Steward. 1952. Coconut milk factor: the growth promoting substances in coconut milk. Jour. Amer. Chem. Soc. 74: 6133-6135.

_____ and _____. 1955. The identification of compound A from coconut milk as 1,3-diphenylurea. Jour. Amer. Chem. Soc. 77: 6351-6353.

Shushan, Sam. 1959. Developmental anatomy of an orchid, Cattleya Trimos. In The Orchids. Carl Withner, ed. Ronald Press, New York. Pp. 45-72.

Wimber, D. E. 1963. Clonal multiplication of Cymbidiums through tissue culture of the shoot meristem. Amer. Orchid Soc. Bull. 32:105-107.

_____. 1965. Additional observations on clonal multiplication of Cymbidium through culture of shoot meristems. Cymbidium Soc. News 20(4):7-10.

Withner, C. L. 1953. Germination of "Cyps". Orchid Jour. 2(10):473-477.